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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Claude	Maina	West Newbury, MA
Jianping	Xiao	Wenham, MA
George	Tzertzinis	Cambridge, MA

Additional inventors are being named on the 1 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Compositions and Methods for Generating Short Double-Stranded RNA Using Mutated RNase III

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

[X] Customer Number: 28986

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ENCLOSED APPLICATION PARTS *(check all that apply)*

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Respectfully submitted,

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Date 01/23/04

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REGISTRATION NO. 37,008 *(If appropriate)*

Docket Number: NEB-238

TELEPHONE 978-927-5054

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Docket Number NEB-238

INVENTOR(S)/APPLICANT(S)		
Given Name (first and middle [if any])	Family or Surname	Residence (City and either State or Foreign Country)
Larry	McReynolds	Beverly, MA

Number 1 of 1

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Practitioner's Docket No. NEB-238 *PATENT*

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Maina, Claude; Xiao, Jianping; Tzertzinis, George; McReynolds, Larry

Application No.: not yet assigned Group No.: N/A
Filed: herewith Examiner: N/A
For: Compositions and Methods for Generating Short Double-Stranded RNA Using Mutated RNase III

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COMPOSITIONS AND METHODS FOR GENERATING SHORT DOUBLE-STRANDED RNA USING MUTATED RNASE III

Inventors: Claude Maina, Jianping Xiao, George Tzertzinis and Larry McReynolds

THIS IS A PROVISIONAL APPLICATION

BACKGROUND OF THE INVENTION

RNA interference (RNAi) employing short double-stranded RNA (siRNA) is a powerful tool for silencing gene expression (WO 01/29058, WO 01/68836, WO 01/75164). Large fragments of double-stranded RNA (dsRNA) elicit a non-specific response in mammalian cells through activation of the interferon (IFN) response pathway that leads to suppression of translation and cell death (Yang, et al., *Mol. Cell. Biol.* 21:7807-7816 (2001) and Wianny, et al., *Nat. Cell Biol.* 2:70-25, 25-33 (2000)). The standard method for generating siRNA is based on chemical synthesis of a pre-determined short sequence. In addition to the high cost of this method, there is no known method for predicting the short sequences effective for RNAi experiments and the users of the method resort to a "trial and error" approach.

A mixture of short lengths of double-stranded RNA obtained through partial digestion of long dsRNA with RNase III in the presence of magnesium ion buffer has been shown to

"knock-down" the expression of cognate genes in cultured mammalian cell lines via RNAi (Yang, et al., *Proc. Nat'l. Acad. Sci. USA* 99:9942-9947 (2002)). However, achieving partial digestions yielding the right size range of product is often a difficult and time-consuming process and requires gel separation to obtain fragments of the desired size. Furthermore the inclusion of all possible sequences contained in the starting material is not ensured. US Patent Application No. 10-622240 herein incorporated by reference, describes how RNAse III in the presence of transition metal ions can produce a heterogeneous mixture of fragments of a size suitable for gene silencing. This is a significant improvement on existing methods of making siRNA fragments. However, it would be desirable to circumvent the reliance on transition metal ions for forming siRNA mixtures enzymatically.

Brief Description of the Figures

Figure 1 shows a chart containing amino acid sequences important for cleavage by bacterial RNAse III (top panel) and putative corresponding regions in the two domains for Dicer enzymes in the lower two panels. In particular, mutations were targeted to amino acids (38, 45, 65 and 117) in *E. coli* RNAse III corresponding to amino acids numbered 37, 44, 64 and 110 in RNaseIII in A. aeolicus. Nine RNaseIII mutants are listed.

Figure 2 shows the activity of E38A protein:

A. Digestion of 500 ng of ds MalE dsRNA (900bp) with a serial dilution of E38A mutant RNase III in Mg^{2+} buffer (4, 2, 1,

0.125 micrograms) (lane d-a). Digestions were conducted at 37°C for 60 mins. Lane (b) with 1ug of enzyme shows the smallest ratio of enzyme to substrate where complete digestion is observed. Lane (d) shows that the amount of 23 bp product is ~60% of the ds RNA substrate.

B Digestions were set up as in A but were incubated overnight, demonstrating enhanced stability of the 23 bp product over an extended period of time in the presence of the complete RNaseIII containing mixture.

C. The cartoon depicts a current model of the digestion of ds RNA with RNAse III. The enzyme is a dimer with amino acids shown that are believed to contact the ds RNA and to be responsible for cleavage (Structure, 9, 1225-1236, 2001).

Figure 3 shows a time course reaction using 4 micrograms of E38A per 500 ngs of 900 bp MalE ds RNA in NEB Buffer 2 (50mM NaCl, 10mM TRIS HCL, 10mM $MgCl_2$, 1mM DTT pH 7.9). Samples were removed at times indicated. The 23 bp product is still present even after 5 days of digestion. This stability is greater than that observed for WT RNAse III. Right most lane is a mock digestion.

Figure 4 shows how E65A which has been purified and assayed under reaction conditions used for E38A produced a 23 bp product which was less stable than for E38A and did not survive overnight digestion. Both mutants produced an increased yield of the 23 bp product as compared to wild type RNase III in Mg buffer.

Figure 5 provides a summary of enzyme activity for different mutants. E38Q, E65P, and the double mutant E38Q/E65P all had activity similar to WT RNAse III. D45V and the double mutant E38A/E65A appear to produce the same product as WT RNase III but at a much slower rate. D45A and E117D have no activity. E38A and E65A produce a 23 bp product.

DESCRIPTION OF THE EMBODIMENTS

We report the selective generation of short dsRNA effective in silencing gene expression using digestions with RNase III mutants in the presence of standard buffers containing magnesium. Different types of mutants are described: single point mutations altering RNA binding or cleavage residues, double point mutants and domain composite enzymes using modules of wild type or mutant enzyme sequences. Examples of mutants are provided that have comparable activity to that described for wild type RNase III in a manganese containing buffer and described in US Patent Application No. 10-622240.

The advantages of RNase III mutants such as E38A described herein include: (a) the ability to obtain the desirable size range of double-stranded RNA products generated by substantially complete digestion of larger dsRNA molecules corresponding to a large portion or the total sequence of the target mRNA so as to circumvent the need for selecting an effective target short sequence (b) incubation in standard buffers to facilitate making ds RNA and cleaving it to the desired size all

in a single reaction vessel; (c) enhanced yield of fragments in the desired size range; and (d) enhanced stability of the fragments obtained using mutant enzymes compared with wild type RNase III in a standard Magnesium buffer.

The fragmentation of large dsRNA molecules (greater than 100 bp) provides a population of short RNAs which include multiple effective short sequences (20-25bp) corresponding to the target RNA for silencing. In a preferred embodiment, the short RNAs represent at least 30% of the total digest and may represent as much as 40% or 50%.

Additionally, the advantages of the method described herein obviate the need for calibration of the time of digestion or the amount of enzyme used, and further eliminate the need to remove undesired digestion products by gel electrophoresis or other tedious separation methods making the method amenable to automation and suitable for high throughput formats. The RNA starting material can be readily obtained by *in vitro* enzymatic transcription or chemical synthesis and can be a double-stranded molecule or a hairpin.

The size range of the RNase digested dsRNA products made according to the methods of the invention is in the range of 18-50 bp more particularly 20-25 bp, more particularly 21-23bp suitable for RNA silencing in cultured mammalian and insect cells. (Herein the fragment size is described as about 23bp where this size does not exclude fragments in the range of fragments within 20-25bp). It is expected that these fragments

will also be active in RNAi silencing in whole organisms such as, plants, microorganisms and animals including humans as well as to cultured cells from the same.

The digestion of double stranded RNA preferably results in at least 30% of the preparation having the desired fragment size. More particularly, the desired percentage achieved with mutant RNAse III is greater than 40% more particularly greater than 50%. The size range of the RNase digested dsRNA products made according to the methods of the invention is suitable for RNA silencing in cultured mammalian and insect cells. It is expected that these fragments will also be active in RNAi silencing in whole organisms such as, plants, microorganisms and animals including humans as well as to cultured cells from the same.

All references cited herein are incorporated by reference.

EXAMPLES

Example I: Preparation of E.coli RNase III mutants

All E. coli RNAse III mutants (except for E117D which was generated by random mutagenesis using PCR) were constructed by a standard 2 step PCR sewing technique (Methods Enzymol. 185, 60-89, 1990). The starting plasmid was E. coli RNAse III cloned into pET16B which produces a His-tagged RNAse III protein under control of a T7 promoter.

WT RNAse III

E. coli RNAse III was amplified from a pMalE/RNAse III clone with the following primers:

SEQ 1 - ACAGGATCCCATGAACCCCATCGTAATTAAT

SEQ 2 –ACAGGATCCTCTAGAGTCATTCCAGCTCCAGTTTTT

The PCR product was cleaved with BamHI and cloned into the BamHI site of pET16b, resulting in a plasmid that synthesizes His-tagged WT RNAse III.

Formation of E38A mutant

The primers used to construct the carboxy terminal half of *E.coli* RNase III (Accession No. X02946) with an E38A mutation were: CAGTAAACATAACGCGCGTTTAGAAT and primer SEQ 2-ACAGGATCCTCTAGAGTCATTCCAGCTCCAGTTTTT

The primers used to construct the amino terminal half of RNAse III with an E38A mutation were: SEQ 3 AATTCTAAACGCGCGTTATGTTTACT and NEB primer cat# 1248.

The two PCR products were then 'sewn' together in one PCR reaction using both as substrates and NEB primers 1228 & 1248 as primers for the reaction. The resulting product was then cloned back into pET16b at a XbaI site, resulting in a His-tagged RNAse III with an E38A mutation.

Formation of E65A RNase III

E65A RNAse III was constructed in a two-step process. In the first step the above plasmid was amplified with the following primers in two PCR's:

SEQ 4 – TCCGGCTCATATCGCCTGCATCCACACGAGGGA

SEQ 2- ACAGGATCCTCTAGAGTCATTCCAGCTCCAGTTTTT

SEQ 5 – CTCGTGTGGATGCAGGCGATATGAGCCGGAT

The two PCR products were then used as substrates in a subsequent PCR reaction with the following primers:

Primer NEB cat No. 1248 and SEQ 2-

ACAGGATCCTCTAGAGTCATTCCAGCTCCAGTTTTT

The resulting PCR product was cleaved with XbaI and cloned into the XbaI site of the modified pET16b vector described in I.B.

Pseudo-Dicer (0 spacing)

The dsRNA cleavage domain of E. coli RNAse III was amplified from a His-tagged WT RNAse III clone with the following primers from NEB cat No. 1248:

SEQ 6: ACACATATGATCTTTTTGTTTATCGCCTGGGCTAAT

SEQ 7: ACACATATGAACCCCATCGTAATTAATCGGCT

The PCR product was cleaved with Nde I and cloned into the NdeI site of His-tagged E117D RNAse III plasmid. The resulting plasmid, Pseudo-Dicer (0 spacing), was used for activity assays and for further Pseudo-Dicer constructions.

Pseudo-Dicer; 59, 157, 271 spacing

cDNA clones of *S. pombe* Dicer, *B. malayi* Dicer (D. Spiro) and human Dicer were amplified with the following set of primers.

S. pombe

294-247 - ACACTCGAGGGACTTGACTCAGCACTCAAGAT

293-290 - ACACTCGAGTTTCTTGTTTTTAAATGAATAT

B. malayi

295-226 - ACACTCGAGAAGTTCATTGAGAATGTCCTTGA

295-227 - ACACTCGAGCCGATCATGGAAACGATAGCCA

Human

305-099 -ACACTCGAGTATGAAGATGATTTCCTGGAGTATGAT

304-199 -ACACTCGAGCTTATTCTTGAATCTGTAGTTGAT

PCR products were cut with Xho I, and cloned into the Xho I site of His-tagged Pseudo-Dicer (0 spacing) described in II.B.

Example 2: Production and purification of RNase III mutants

Expression & Purification.

30 ml cultures of each mutant and WT clones were grown in E. coli ER2566 (NEB) to mid log phase, then induced by the addition of IPTG to a final concentration of 100 mM and shaken at 15°C overnight. Induced cultures were lysed by sonication.

The RNaseIII mutants were purified from the cleared lysates by Qiagen Nickel resin affinity purification (according to Manufacture's instructions) and quantitated by standard methods. The enzyme reaction was performed in NEB Buffer 2, at 37°C, for 1 hr using 500 ngs of a 900 bp ds RNA as a substrate. The product of the reaction was analyzed by polyacrylamide electrophoresis.

RNase Activity Assay

1 ug of MalE dsRNA was used digested with 1 ml of each of the RNAse III (wt and mutants in the range consistent with optimal conditions shown in Figure 2) in a 20 ml reaction

mixture at 37°C in NEB Buffer 2 (0.1 M NaCl, 50 mM Tris-HCl, pH 7.9, 10 mM $MnCl_2$, 1 mM dithiothreitol) for 1 hour.

As can be seen in the Figure 2, WT RNAse III digests the dsRNA to a size too small to be resolved on a 20% acrylamide gel. However, both the E38A and E65A mutants, in a 1 hour digestion, show a prominent band that co-migrates with a 23 dsRNA size marker. After an overnight digestion, this prominent band disappears from the E65A digestion but still remains in the E38 digestion.

Both the E38A and E65A RNAse III mutants show a dsRNA product of a predicted size of 23 bps after a 1 hour digestion. After an overnight digestion this product still remains for the E38A mutant. The activity of E65A is in direct contrast to what was reported in (Structure, 9, pp1225-1236, 2001). In this paper the authors describe an E65A mutation of RNAse III as disabling RNAse III function. Although the authors do not specifically discuss an E38A mutation of RNase III, they do describe an E38V mutation as also rendering RNase III inactive and stating further that E38 is essential for RNase III activity. Since alanine, like valine, is very much different than the wild type residue glutamic acid, it would be reasonably assumed that an E38A mutation of RNase III would also be inactive. This is not what we observe. The activity of E38A is indeed not wild type but it is not inactive either. Its activity on long dsRNA is to produce a dsRNA product of about 23 bps in length.

Using 52,000 kD for MW of a E38A holoenzyme and 650 kD per bp of dsRNA, the ratio of enzyme to substrate is 23/1 (Lane b) or 1 enzyme per 39 bps. The amount of 23 bp product shown in lane D is ~60% of the ds RNA substrate. This is about twice the yield as that with WT RNAse III with Mn^{2+}.

Of the mutants listed in Figure 1, E117D had no dsRNA cleavage activity. E38Q and E38Q/E65P, shown in black had dsRNA cleavage activity similar to wt. E38Q/E65P had dsRNA cleavage activity similar to wt. E65A and E38A had improved activity over wild type.

Example III: DsRNA cleavage and RNAi Activity in cultured cells.

. RNAi Activity

To test the ability of the dsRNA product of RNase III mutant digestion to induce RNA interference-the following experiment is performed. dsRNA made from firefly luciferase is cleaved with the E38A or E65A mutant RNaseIIIs (Figure 1); the dsRNA product is then purified by ethanol precipitation. Drosophila Schneider S2 cells are transfected with a reporter plasmid expressing the firefly luciferase gene, another reporter acting as a transfection control and dsRNA. Cells transfected with the reporter and no dsRNA will show significant luciferase activity. Cells transfected with the reporter and full-length (approximately 1kB) luciferase dsRNA will show a decrease in luciferase activity. There is no effect when GFP dsRNA is used and there is a significant decrease in luciferase activity when the luciferase dsRNA cleaved by the RNase III mutant is used.

What is claimed is:

1. An RNAse III mutant, characterized by its ability to cleave a double stranded RNA into fragments of about 23bp such that at least 30% of the preparation is in the form of 23bp fragments, the cleavage occurring in the presence of magnesium ions.

2. An RNase III mutant according to claim 1, characterized by at least one point mutation located in the region associated with cleavage.

3. An RNase III mutant according to claim 2, wherein the at least one point mutation comprises a mutation at position 38 of the protein.

4. An RNase III mutant according to claim 2, wherein the at least one point mutation comprises a mutation at position 65 of the protein.

5. An RNase III mutant according to claim 2, wherein the RNase III is derived from *E.coli*.

6. Dicer purified as in Example II and tested as in Example III

E. coli RNAse III Mutants

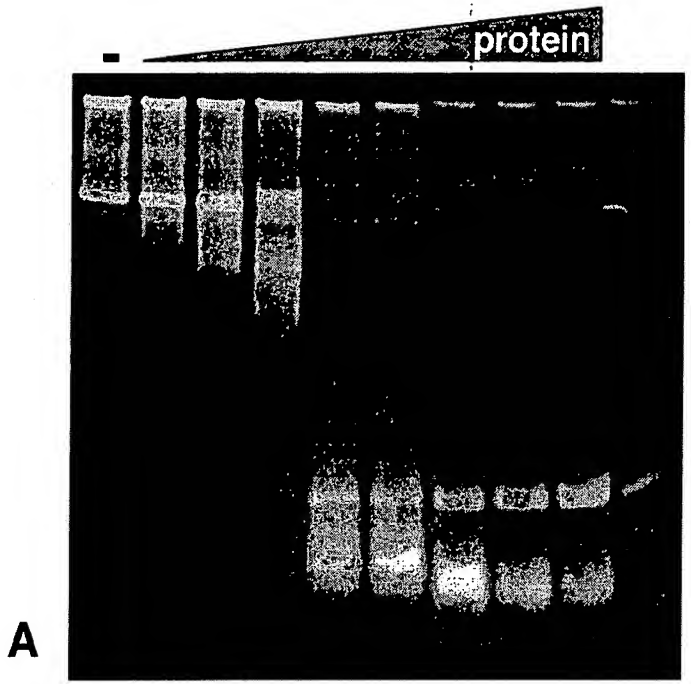
RNAse III

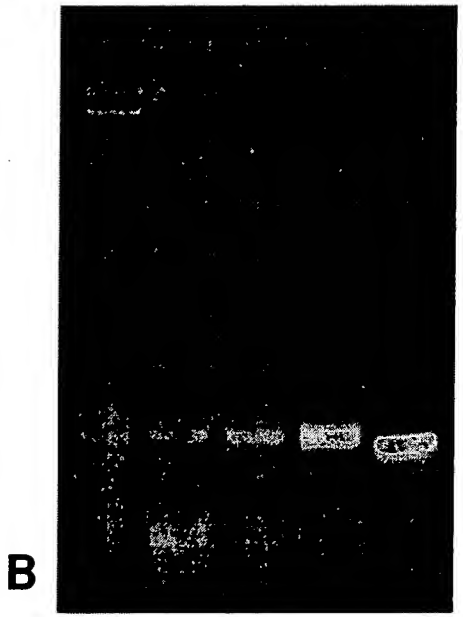
	37	40	44		64			110
Aquifex aeolicus	37	E T L	E F L G D A	63	R E G	F L S	107	D V F E A L
E. coli	38	E R L	E F L G D S	64	D E G	D M S	114	D T V E A L
E38Q	38	Q R L	E F L G D S	64	D E G	D M S	114	D T V E A L
E38A	38	A R L	E F L G D S	64	D E G	D M S	114	D T V E A L
D45A	38	E R L	E F L G A S	64	D E G	D M S	114	D T V E A L
D45V	38	E R L	E F L G V S	64	D E G	D M S	114	D T V E A L
E65P	38	E R L	E F L G D S	64	D P G	D M S	114	D T V E A L
E65A	38	E R L	E F L G D S	64	D A G	D M S	114	D T V E A L
E117D	38	E R L	E F L G D S	64	D E G	D M S	114	D T V D A L
E38Q,E65P	38	Q R L	E F L G D S	64	D P G	D M S	114	D T V E A L
E38A,E65A	38	A R L	E F L G D S	64	D A G	D M S	114	D T V E A L

Dicer

Amino								
Drosha	833	E R L	E F L G D A	859	E E G	G L A	909	N C F E A L
Dm-Dicer	1742	E R L	E T I G D S	1768	H E G	K L S	1818	K E L E K A
Ce-Dicer	1348	E R F	E T I G D S	1374	H E G	K L S	1433	D A E E K D
Hs-Dicer	1315	E R L	E M L G D S	1341	H E G	R L S	1397	D K W E K D
Mm-Dicer	1314	E R L	E M L G D S	1340	H E G	R L S	1396	E K W E K D
Sp-Dicer	930	D R L	E F Y G D C	956	Q E Y	Q L H	1024	D M V E A S
Ag-Dicer	1173	E R F	E V L G D A	1199	H E G	Y L T	1257	D A N E S P
Nc-Dicer	998	E R L	E F I G D T	1031	H E R	L L S	1099	D V V E S L
B. malayi-Dicer	1428	E R L	E T V G D S	1454	H E G	K L S	1504	S D F K A P
Carboxy								
Drosha	1012	Q R L	E F L G D T	1038	H E G	H L S	1084	D L L E A F
Dm-Dicer	2029	Q R L	E F L G D A	2055	S P G	A L T	2136	D V F E S I
Ce-Dicer	1614	Q R L	E F L G D A	1640	S P G	V L T	1726	D I F E S V
Hs-Dicer	1704	Q R L	E F L G D A	1730	S P G	V L T	1812	D I F E S L
Mm-Dicer	1697	Q R L	E F L G D A	1723	S P G	V L T	1805	D I F E S L
Sp-Dicer	1120	Q Q L	E F L G D A	1146	T S G	E L T	1219	D T L E A M
Ag-Dicer	1417	Q Q L	E F L G D A	1443	S P G	Q L T	1525	D V L E A L
Nc-Dicer	1196	E R L	E F L G D A	1224	A H N	D M H	1345	D I V E S L
B. malayi-Dicer	1698	Q R L	E F L G D A	1724	S P G	V L T	1811	D I F E S V

RNAse Activity of E38A





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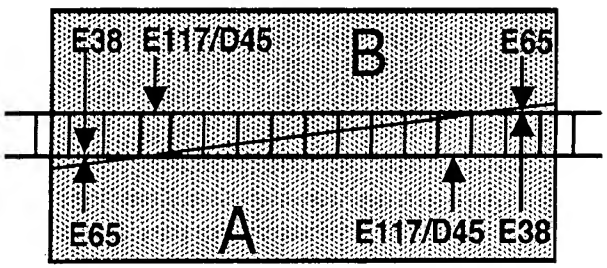
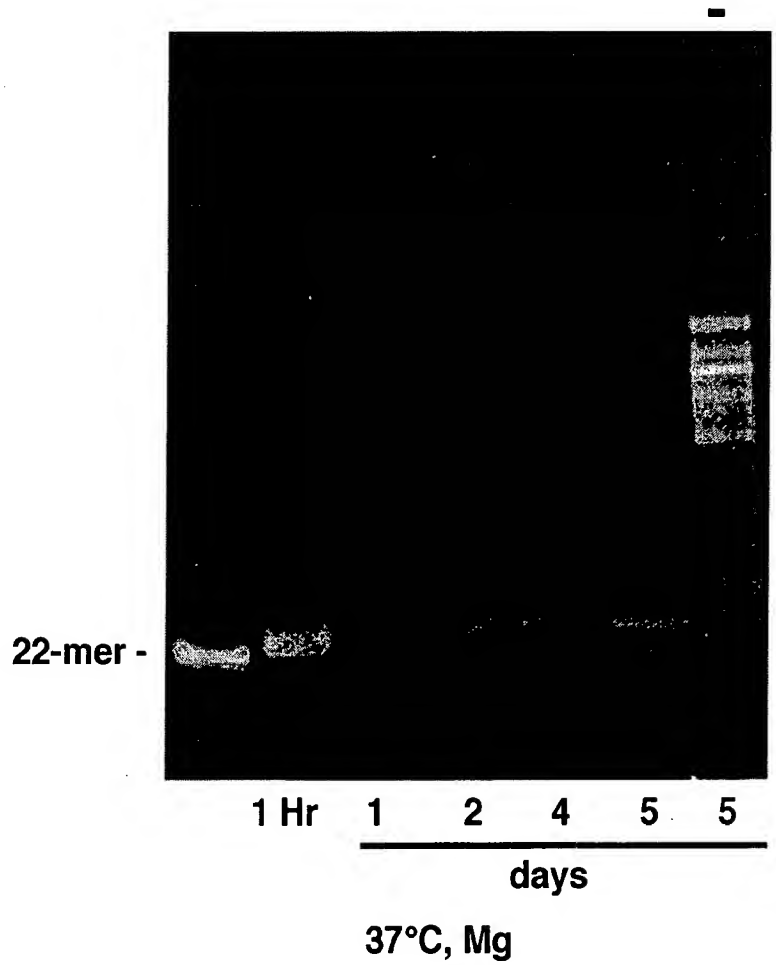


b - 23/1; 1/39 bps

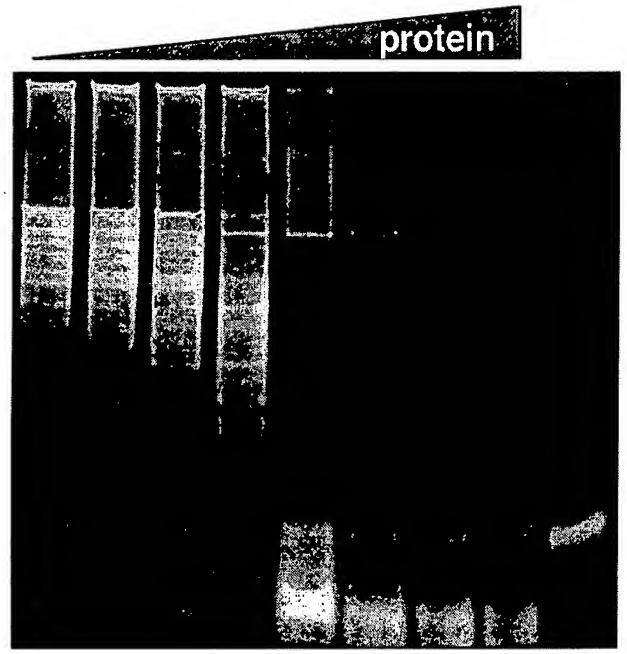
Yield - ~ 60% (d)

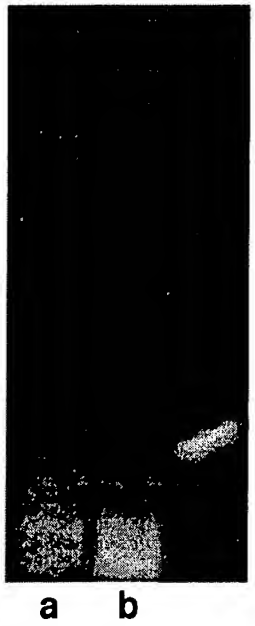
Figure 3

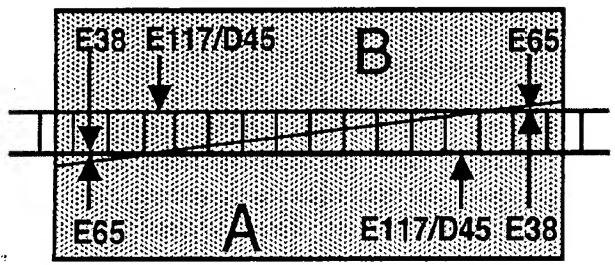
RNAse Activity of E38A



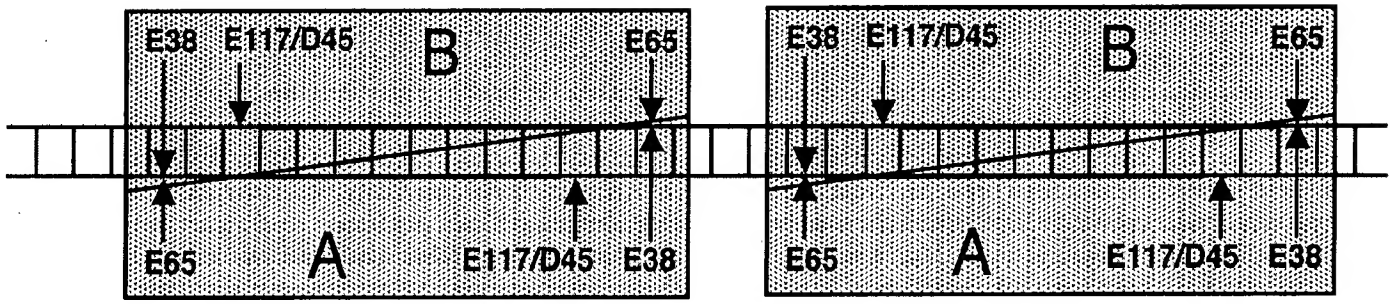
RNAse Activity of E65A







Summary of Mutant Analysis



WT	No Activity	23-mer
E38Q	D45A	E38A
D45V*	E117D	E65A
E65P		
E38A, E65A*		
E38Q, E65P		